(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 May 2004 (27.05.2004)

PCT

(10) International Publication Number
WO 2004/043406 A2

(51) International Patent Classification[7]: A61K

(21) International Application Number:
PCT/US2003/036367

(22) International Filing Date:
12 November 2003 (12.11.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/425,897 12 November 2002 (12.11.2002) US

(71) Applicant: **THE JOHNS HOPKINS UNIVERSITY** [US/US]; 100 N. Charles Street, 5th Floor, Baltimore, MD 21201 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **DEWEESE, Theodore, L.** [US/US]; 7108 Bristol Road, Baltimore, MD 21212 (US). **COLLIS, Spencer, J.** [GB/US]; 3900 N. Charles, Apt. 1017, Baltimore, MD 21218 (US). **NELSON, William, G.** [US/US]; 2006 Ridgecrest Court, Towson, MD 21204 (US).

(74) Agents: **ROSENFIELD, Jennifer, K.** et al.; Edwards & Angell, LLP, P.O. Box 9169, Boston, MA 02209 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/043406 A2

(54) Title: ENGINEERED RNAi ADENOVIRUS SILENCING EXPRESSION (ERASE) OF DNA REPAIR PROTEINS

(57) Abstract: The present invention relates to compositions and methods for killing cancer cells and treating subjects having cancer comprising administering small inhibitory RNAs (siRNA) specific for DNA repair proteins in conjunction with administration of conventional DNA-damaging agents such as radiation and chemotherapeutic agents.

signaling/repair factors ATM, ATR, and DNA-PKcs combined with localized conformal radiotherapy or systemic delivery of chemotherapeutic drugs would make an attractive adjuvant gene therapy approach for many solid tumors. Accordingly, there is a need in the art for methods which can effectively inhibit these kinases in conjunction with DNA-damaging agents.

Summary of the Invention

The present invention is based, at least in part, on the discovery that inhibition of DNA repair protein expression through the use of small inhibitory RNA (siRNA) can augment radiation and chemotherapy-mediated killing of cancer cells by sensitizing the cells to the DNA-damaging action of those therapies. Accordingly, the present invention provides methods for killing cancer cells and methods of treating subjects having cancer, comprising administering siRNAs directed to DNA repair proteins (e.g., ATM and ATR and DNA-PK_{cs}) in combination with one or more DNA-damaging agent (e.g., radiation or a chemotherapeutic agent).

In one embodiment, the invention provides methods of killing tumor cell comprising contacting the cells with at least one small inhibitory RNA (siRNA) specific for a DNA repair protein and at least one DNA-damaging agent. In a preferred embodiment, the DNA repair protein is ATM, ATR, or DNA-PK_{cs}. In another preferred embodiment, the siRNA is encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 30, 31, 32, 33, 34, 35, or 36.

In one embodiment, the DNA-damaging agent is radiation. In another embodiment, the DNA-damaging agent is a chemotherapeutic agent, for example, an alkylating agent such as a nitrogen mustard (e.g., chlorambucil, cyclophosphamide, estramustine, ifosfamide, mechlorethamine, or melphalan), an aziridine (e.g., thiotepa), an alkyl sulfonate (e.g., busulfan), a nitrosurea (e.g., carmustine, lomustine, or streptozocin), a platinum complex (e.g., carboplatin or cisplatin), and a nonclassic alkylator (e.g., altretamine, dacarbazine, procarbazine, or temozolamide).

In another embodiment, the methods of the invention further comprise contacting the cell with at least a second chemotherapeutic agent.

In one embodiment, the tumor cells are derived from a cell or tissue type selected from the group consisting of prostate, colon, breast, lung, brain, skin, ovary, pancreas, liver, stomach, bladder, bone, testicle, uterus, adipose tissue, throat, kidney, tongue, pituitary gland,

sequence, an adenoviral VA1 A-Box, at least one restriction enzyme recognition site, at least one adenoviral VA1 termination sequence, and at least one adenoviral VA1 B-box.In one embodiment, the siRNA is administered to the subject systemically. In another embodiment, the siRNA is administered to the subject locally at the site of a tumor.

In another embodiment, the siRNA is administered prior to administration of the DNA-damaging agent. In another embodiment, the siRNA is administered at the same time as the DNA-damaging agent. In yet another embodiment, the DNA-damaging agent is administered prior to the siRNA.

In still another embodiment, the invention provides an isolated nucleic acid molecule comprising a modified adenoviral VA1 promoter, wherein the modified adenoviral promoter comprises, in sequence, an adenoviral VA1 A-Box, at least one restriction enzyme recognition site, at least one adenoviral VA1 termination sequence, and at least one adenoviral VA1 B-box.

In another embodiment, the invention provides nucleic acid molecules comprising the nucleic acid sequence of SEQ ID NO:1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 30, 31, 32, 33, 34, 35, or 36.

Other features and advantages of the invention will be apparent to those skilled in the art from the following detailed description and claims.

Brief Description of the Drawings

Figures 1A-F depicts siRNA-mediated down-regulation of ATM, ATR, and DNA-PK_{cs} proteins in DU 145 cells after 48-hour transfection with siRNA-encoding plasmids. Figures 1A, 1C, and 1E, Western blots for ATM, ATR, and DNA-PK_{cs}, respectively. Membranes were probed with antibodies for target protein, and expression levels were normalized for loading by probing for β-actin. Protein expression for ATM, ATR, and DNA-PK_{cs} was quantified using a BioRad Versa-Doc imager and Quantity One analysis software and expressed as a percentage compared with that calculated in untransfected cells (Figures 1B, 1D, and 1F, respectively). Given that the respective transfection efficiencies were calculated as ~45, 40, and 50, ≤90% inhibition of target protein are evident in the transfected population.

Figures 2A-2D depict clonogenic survival of DU 145 and PC-3 cells after transient transfection with ATM and DNA-PK_{cs}-targeted, siRNA-encoding plasmids followed by

Figure 9 depicts the effectiveness of the VA1 promoter in DU 145 cells in cotransfection experiments.

Figure 10 depicts the results of VA1 versus U6-mediated downregulation of DNA-PK in DU 145 cells.

Figure 11 depicts the results of VA1 versus U6-mediated downregulation of DNA-PK in DU 145 cells at 72 hours post-transfection.

Figure 12 depicts the results of VA1-mediated downregulation of DNA-PK in DU145 cells at 72 hours post-transfection.

Figure 13 depicts the results of VA1 versus U6-mediated downregulation of DNA-PK in DU 145 cells at 72 and 96 hours post-transfection.

Figure 14 depicts the sequences of the oligonucleotides used to construct the ATM-1 siRNA-encoding nucleic acid molecules. Oligo-A: SEQ ID NO:1. Oligo-B: SEQ ID NO:2. Target sequence: SEQ ID NO:3.

Figure 15 depicts the sequences of the oligonucleotides used to construct the ATM-2 siRNA-encoding nucleic acid molecules. Oligo-A: SEQ ID NO:4. Oligo-B: SEQ ID NO:5. Target sequence: SEQ ID NO:6.

Figure 16 depicts the sequences of the oligonucleotides used to construct the ATM-3 siRNA-encoding nucleic acid molecules. Oligo-A: SEQ ID NO:7. Oligo-B: SEQ ID NO:8. Target sequence: SEQ ID NO:9.

Figure 17 depicts the sequences of the oligonucleotides used to construct the ATR-1 siRNA-encoding nucleic acid molecules. Oligo-A: SEQ ID NO:10. Oligo-B: SEQ ID NO:11. Target sequence: SEQ ID NO:12.

Figure 18 depicts the sequences of the oligonucleotides used to construct the ATR-2 siRNA-encoding nucleic acid molecules. Oligo-A: SEQ ID NO:13. Oligo-B: SEQ ID NO:14. Target sequence: SEQ ID NO:15.

Figure 19 depicts the sequences of the oligonucleotides used to construct the ATR-3 siRNA-encoding nucleic acid molecules. Oligo-A: SEQ ID NO:16. Oligo-B: SEQ ID NO:17. Target sequence: SEQ ID NO:18.

Figure 20 depicts the sequences of the oligonucleotides used to construct the DNA-PK-1 siRNA-encoding nucleic acid molecules. Oligo-A: SEQ ID NO:19. Oligo-B: SEQ ID NO:20. Target sequence: SEQ ID NO:21.

Figure 21 depicts the sequences of the oligonucleotides used to construct the DNA-PK-2 siRNA-encoding nucleic acid molecules. Oligo-A: SEQ ID NO:22. Oligo-B: SEQ ID NO:23. Target sequence: SEQ ID NO:24.

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Highly specialized DNA repair proteins together with upstream sensors and signalers protect mammalian cells from potentially lethal and/or tumorigenic lesions resulting from DNA damage (for recent reviews, see Jackson, S. P. (2001) Biochem. Soc. Trans. 29:655–661; Hoeijmakers, J. H. (2001) Nature, 411:366–374). Pivotal to these repair pathways are the DNA damage sensors ATM and ATR and DNA-PK_{cs}. The essential role these proteins play in DNA damage and repair is highlighted by the extreme sensitivity to DNA-damaging agents exhibited by cells and animal models defective in and/or lacking ATM, ATR, and DNA-PKcs expression (Miller, S. P. et al. (1995) Science, 267:1183-1185; Meyn, M. S. (1995) Cancer Res., 55: 5991–6001; Shiloh, Y. (2001) Curr. Opin. Genet. Dev. 11:71–77). Thus, targeted inhibition of these kinases is an attractive approach in the development of potent radiation therapy strategies. To increase the radio- and chemotherapy-mediated cell killing of human tumor cells, the present invention uses vector systems, including a plasmid-based pol III promoter system (Paddison, P. J. et al. (2002) Genes Dev. 16:948–958) and an adenoviral vector system, to deliver and express siRNAs targeted toward DNA repair proteins such as ATM, ATR, and DNA-PK_{cs}.

The data presented herein demonstrate the effective use of siRNA as a novel tool for modulating killing of human cancer cells by DNA-damaging agents, including radiation and chemotherapeutic agents. The inherent specificity of this approach provides a powerful method of target protein downregulation that can be incorporated into several existing viral and nonviral vector delivery platforms, including adenoviral vectors.

internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides. For purposes of this invention, adenovirus vectors are replication-competent in a target cell.

As used herein, the term "vector" refers to a polynucleotide construct designed for transduction/transfection of one or more cell types. Vectors may be, for example, "cloning vectors" which are designed for isolation, propagation and replication of inserted nucleotides, "expression vectors" which are designed for expression of a nucleotide sequence in a host cell, or a "viral vector" which is designed to result in the production of a recombinant virus or virus-like particle, or "shuttle vectors", which comprise the attributes of more than one type of vector.

The terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides and/or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be a oligodeoxynucleoside phosphoramidate ($P\text{-}NH_2$) or a mixed phosphoramidate-phosphodiester oligomer. Peyrottes et al. (1996) Nucleic Acids Res. 24: 1841-8; Chaturvedi et al. (1996) Nucleic Acids Res. 24: 2318-23; Schultz et al. (1996) Nucleic Acids Res. 24: 2966-73. A phosphorothioate linkage can be used in place of a phosphodiester linkage. Braun et al. (1988) J. Immunol. 141: 2084-9; Latimer et al. (1995) Molec. Immunol. 32: 1057-1064. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer. Reference to a polynucleotide sequence (such as referring to a SEQ ID NO) also includes the complement sequence.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified

The term "replication" means duplication of a vector. This duplication, in the case of viruses, can occur at the level of nucleic acid, or at the level of infectious viral particle. In the case of DNA viruses, replication at the nucleic acid level comprises DNA replication. In the case of RNA viruses, nucleic acid replication comprises replication into plus or minus strand (or both). In the case if retroviruses, replication at the nucleic acid level includes the production of cDNA as well as the further production of RNA viral genomes. The essential feature is the generation of nucleic acid copies of the original viral vector. However, replication also includes the formation of infectious DNA or RNA viral particles. Such particles may successively infect cells in a given target tissue, thus distributing the vector through all or a significant portion of the target tissue.

In the context of adenovirus, a "heterologous polynucleotide" or "heterologous gene" or "transgene" is any polynucleotide or gene that is not present in wild-type adenovirus. Preferably, the transgene will also not be expressed or present in the target cell prior to introduction by the adenovirus vector.

In the context of adenovirus, a "heterologous" promoter or enhancer is one which is not associated with or derived from an adenovirus gene.

A "host cell" includes an individual cell or cell culture which can be or has been a recipient of an adenoviral vector(s) of this invention. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change. A host cell includes cells transfected or infected in vivo or in vitro with an adenoviral vector of this invention.

A "biological sample" encompasses a variety of sample types obtained from an individual and can be used in a diagnostic or monitoring assay. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom, and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term "biological sample" encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, rodents, primates, and pets

described herein are those who are considered high risk for developing a tumor, such as those who have a genetic predisposition to development of a neoplasia and/or who have been exposed to an agent(s) which is correlated with development of a neoplasia. In one embodiment, the methods of the present invention are particularly useful for the treatment of tumors that are resistant to at least one form of cancer therapy, including radiation and/or chemotherapy.

The presence of cancer and the suitability of the individual for receiving the methods described herein may be determined by any of the techniques known in the art, including diagnostic methods such as imaging techniques, analysis of serum tumor markers, and biopsy.

The siRNA may be targeted to any DNA repair protein known to participate in DNA repair pathways activated in response to DNA-damaging agents, including, but not limited to, ATM, ATR, and/or DNA-PK_{cs}. The siRNA may target any region in the target mRNA, and may be encoded, for example, by one ore more of the nucleic acid sequences set forth in SEQ ID NO:1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 30, 31, 32, 33, 34, 35, or 36. The methods of the invention may use siRNA targeting a single DNA repair protein, or may use a mixture of siRNA targeting more than one DNA repair protein. For example, in a preferred embodiment, the methods of the invention use siRNA targeting one, two, three or more DNA repair proteins.

As used herein, a "DNA-damaging agent" is any agent or treatment that, when administered to a cell or a subject, e.g., a human subject, cause damage to the cell or subject's DNA (e.g., genomic DNA). In one embodiment, the DNA-damaging agent is radiation. In another embodiment, the DNA-damaging agent is a chemotherapeutic agent. Preferred chemotherapeutic DNA-damaging agents include, but are not limited to, alkylating agents such as nitrogen mustards (e.g., chlorambucil, cyclophosphamide, estramustine, ifosfamide, mechlorethamine, and melphalan), aziridines (e.g., thiotepa), alkyl sulfonates (e.g., busulfan and methyl methanesulfonate (MMS)), nitrosureas (e.g., carmustine, lomustine, and streptozocin), platinum complexes (e.g., carboplatin and cisplatin), and nonclassic alkylators (e.g., altretamine, dacarbazine, procarbazine, and temozolamide). In some embodiments, the methods of the present invention comprise the use of one or more DNA-damaging agents.

In another embodiment, the methods of the invention can be administered in conjunction with other known treatments for cancer, including, but not limited to, mechanical removal of cancerous cells (e.g., surgical removal of a tumor), and administration of chemotherapeutic agents. In addition to DNA-damaging agents, there are many other

of the invention will be used in accordance with standard dosage levels and schedules for the DNA-damaging agents. A useful general resource for methods of treating and managing cancer is Pazdur, R. et al., eds., *Cancer Management: A Multidiscliplinary Approach, 7th edition*. The Oncology Group, a division of SCP Communications, Inc., New York:2003. Non-limiting exemplary chemotherapy and radiation treatment regimens may be further found, for example, in Bonadonna, G. et al. (1976) N. Engl. J. Med. 294:405–410; Bonadonna, G. et al., in Salmon SE (ed): Adjuvant Therapy of Cancer VI, pp 169–173, 1990; Weiss, R.B. et al. (1987) Am J Med 83:455–463; Budman, D.R. et al. (1992) Proc. Am. Soc. Clin. Oncol. 11:51; Hortobagyi, G.N. et al. (1979) Cancer 43:1225–1233; Fisher, B. et al. (1990) J. Clin. Oncol. 8:1483; Henderson, I.C. et al. (1998) Proc. Am. Soc. Clin. Oncol. 17:101A; Slamon, D. et al. (1998) Proc. Am. Soc. Clin. Oncol. 17:377A; Norton, L. et al. (1999) Proc. Am. Soc. Clin. Oncol. 18:483A; Pouillart, P. et al. (1999) Proc. Am. Soc. Clin. Oncol. 18:2275A; Nabholtz, J.M. et al. (1999) Proc. Am. Clin. Oncol. 18:485A; Seidman, A.D. et al. (2000) Proc. Am. Soc. Clin. Oncol. 19:319A; Seidman, A.D. et al. (1995) J. Clin. Oncol. 13:2575–2581; Ravdin, P.M. et al. (1995) J. Clin. Oncol. 13:2879–2885; Vogel, C. et al. (2000) Proc. Am. Soc. Clin. Oncol. 19:275A; Michaud, L. et al. (2000) Proc. Am. Soc. Clin. Oncol. 19:402A; Leichman, L. et al. (1985) Am. J. Med. 78:211–215; Wagner, J.P. et al. (1994) Int. J. Radiat. Oncol. Biol. Phys. 29:17–23; Poon, M.A. et al. (1989) J. Clin. Oncol. 7:1407–1418; Moertel, C.G. et al. (1995) Ann. Intern. Med. 122:321–326; Moertel, C.G. et al. (1990) N. Engl. J. Med. 322:352–358; Wolmark, N. et al. (1996) Proc. Am. Soc. Clin. Oncol. 15:205; Moertel, C.G. et al. (1994) J. Clin. Oncol. 12:21–27; Saltz, L.B. et al. (1999) Proc. Am. Soc. Clin. Oncol. 18:898A; Saltz, L.B. et al. (2000) N. Engl. J. Med. 343:905?14; Pitot, H.C. et al. (1997) J. Clin. Oncol. 15:2910–2919; Cunningham, D. et al. (1998) Proc. Am. Soc. Clin. Oncol. 17:1A; Twelves, C. et al. (1999) Proc. Am. Soc. Clin. Oncol. 18:1010A; Herskovic, A. et al. (1992) N. Engl. J. Med. 326:1593–1598; Preusser, P. et al. (1989) J. Clin. Oncol. 7:1310–1317; Lerner, A. et al. (1992) J. Clin. Oncol. 10:536–540; Kelsen, D. et al. (1992) J. Clin. Oncol. 10:541–548; Macdonald, J.S. et al. (2000) Proc. Am. Soc. Clin. Oncol. 19:1A; Gastrointestinal Tumor Study Group (1987) Cancer 59:2006–2010; Moertel, C.G. et al. (1994) J. Clin. Oncol. 12:21–27; Burris, H.A. et al. (1997) J. Clin. Oncol. 15:2403–2413; Sternberg, J.J. et al. (1977) JAMA 238:2282–2287; Loehrer, P.J. et al. (1992) J. Clin. Oncol. 10:1066–1073; Redman, B. et al. (1997) Proc. Am. Soc Clin. Oncol. 16:325A; Kaufman, D. et al. (1998) Proc Am. Soc. Clin. Oncol. 17:320A; Burch, P.A. et al. (1999) Proc. Am. Soc. Clin. Oncol. 18:1266A; Vaishampayan, U. et al. (1999) Proc. Am. Soc. Clin .Oncol. 18:1282A; Pienta, K.J. et al. (1994) J. Clin. Oncol. 12:2005–2012; Tannock, I.F. et

The methods of the invention are intended to be used for any type of tumor, cancer, and/or neoplasm, including, but not limited to, those derived from prostate, colon, breast, lung, brain, skin, ovary, pancreas, liver, stomach, bladder, bone, testicle, uterus, adipose tissue, throat, kidney, tongue, pituitary gland, thyroid, nerve, lymphoid tissue, eye, and/or cervix. Additionally, the methods of the invention are intended to be used for tumors which may be a mixture of more than one cell type, as well as for metastasized tumors which are originally derived from one cell type, but have migrated to a different part of the body.

Although methods of tumor suppression are exemplified in the discussion below, it is understood that the alternative methods described above are equally applicable and suitable, and that the endpoints of these methods (e.g., efficacy of treatment) are measured using methods standard in the art, including the diagnostic and assessment methods described above.

Delivery of adenoviral vectors is discussed infra and is generally accomplished by either site-specific injection (local administration) or intravenously (systemic administration). Direct intra-tumor injections are preferred. Site-specific injections of either vector may include, for example, injections into the portal vein of the liver as well as intraperitoneal, intrapleural, intrathecal, intra-arterial, intra-tumor injections or topical application. These methods are easily accommodated in treatments using adenoviral vectors.

The adenoviral vectors may be delivered to the target cell in a variety of ways, including, but not limited to, liposomes, general transfection methods that are well known in the art (such as calcium phosphate precipitation or electroporation), direct injection, and intravenous infusion. The means of delivery will depend in large part on the particular adenoviral vector (including its form) as well as the type and location of the target cells (i.e., whether the cells are to be transfected or transformed in vitro or in vivo). If used as a packaged adenovirus, adenovirus vectors may be administered in an appropriate physiologically acceptable carrier at a dose of about 1 to about 10. The multiplicity of infection will generally be in the range of about 0.001 to 100. If administered as a polynucleotide construct (i.e., not packaged as a virus) about 0.01 μg to about 1000 μg of an adenoviral vector can be administered. The adenoviral vector(s) may be administered one or more times, depending upon the intended use and the immune response potential of the host, and may also be administered as multiple, simultaneous injections. If an immune response is undesirable, the immune response may be diminished by employing a variety of immunosuppressants, so as to permit repetitive administration, without a strong immune

nucleic acid. For example, with regards to genomic DNA, the term 'isolated' includes nucleic acid molecules which are separated from the viral DNA or chromosome with which the genomic DNA is naturally associated. Preferably, an 'isolated' nucleic acid is free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated siRNA-encoding nucleic acid molecules can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the DNA repair protein that the siRNA targets. Moreover, an 'isolated' nucleic acid molecule can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention, e.g., a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 30, 31, 32, 33, 34, 35, or 36, or a portion thereof, can be constructed using standard molecular biology techniques and the sequence information provided herein.

Moreover, a nucleic acid molecule encompassing all or a portion of SEQ ID NO:1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 30, 31, 32, 33, 34, 35, or 36, or 3 can be isolated by the polymerase chain reaction (PCR) using synthetic oligonucleotide primers designed based upon the sequence of NO:1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 30, 31, 32, 33, 34, 35, or 36.

In a preferred embodiment, the siRNAs used in the methods of the invention are produced by inserting a double-stranded DNA molecule that encodes the siRNA into an expression vector (e.g., a plasmid or an adenoviral vector), such that the siRNA can be expressed in a cell (e.g., a cancer cell). In one embodiment, the siRNAs are encoded by the nucleic acid sequence of SEQ ID NO:1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 30, 31, 32, 33, 34, 35, or 36 (see Figures 14-23). In a preferred embodiment, the siRNAs used in the methods of the invention are encoded by SEQ ID NOs:28-36 (Figure 23), wherein 'X' may be any nucleotide (e.g., A, T, C, or G), and 'X_n' represents a flexible number of nucleotides, wherein 'n' may be any number, provided that the nucleotides represented by 'X_n' are capable of forming a loop in the siRNA hairpin structure. In a preferred embodiment, n = 8.

In another embodiment, an isolated nucleic acid molecule of the invention is a modified VA1 promoter, which comprises, in sequence, an adenoviral VA1 A-Box, at least

95% of the nucleotides of the other strand, usually at least about 98%, and more preferably from about 99 to about 100%. Complementary polynucleotide sequences can be identified by a variety of approaches including use of well-known computer algorithms and software.

In still another embodiment, an isolated nucleic acid molecule of the present invention comprises a nucleotide sequence which is at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or more identical to the nucleotide sequence shown in SEQ ID NO:1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 30, 31, 32, 33, 34, 35, or 36 (e.g., to the entire length of the nucleotide sequence), or a portion or complement of any of these nucleotide sequences. In one embodiment, a nucleic acid molecule of the present invention comprises a nucleotide sequence which comprises part or all of SEQ ID NO:1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 30, 31, 32, 33, 34, 35, or 36, or a complement thereof, and which is at least (or no greater than) 25, 30, 50, 75, 100 or more nucleotides (e.g., contiguous nucleotides) in length.

To determine the percent identity of two nucleic acid or amino acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence (for example, when aligning a second sequence to a nucleotide sequence having 100 nucleotides, at least 30, preferably at least 40, more preferably at least 50, even more preferably at least 60, and even more preferably at least 70, 80, or 90 nucleotides are aligned). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment,

should be identical, or differ by no greater than 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 bases when compared to a sequence disclosed herein or to the sequence of a naturally occurring variant. Such probes can be used as a part of a diagnostic test kit for identifying cells or tissue which contain the expression construct, or which express the expressible sequence .

In another embodiment, nucleic acid molecules of the invention can comprise variants of the sequence elements disclosed herein. Nucleic acid variants (e.g., variants of the siRNA sequence used to target specific DNA repair protein mRNA) can be naturally occurring, such as allelic variants (same locus), homologues (different locus), and orthologues (different organism, e.g., mouse) or can be non-naturally occurring. Non-naturally occurring variants can be made by mutagenesis techniques, including those applied to polynucleotides, cells, or organisms. The variants can contain nucleotide substitutions, deletions, inversions and insertions. Allelic variants result, for example, from DNA sequence polymorphisms within a population (e.g., the human population).

As used herein, the term 'hybridizes under stringent conditions' is intended to describe conditions for hybridization and washing under which nucleotide sequences that are significantly identical or homologous to each other remain hybridized to each other. Preferably, the conditions are such that sequences at least about 70%, more preferably at least about 80%, even more preferably at least about 85% or 90% identical to each other remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley & Sons, Inc. (1995), sections 2, 4, and 6. Additional stringent conditions can be found in Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989), chapters 7, 9, and 11. A preferred, non-limiting example of stringent hybridization conditions includes hybridization in 4 X sodium chloride/sodium citrate (SSC), at about 65-70°C (or alternatively hybridization in 4X SSC plus 50% formamide at about 42-50°C) followed by one or more washes in 1X SSC, at about 65-70°C. A preferred, non-limiting example of highly stringent hybridization conditions includes hybridization in 1X SSC, at about 65-70°C (or alternatively hybridization in 1X SSC plus 50% formamide at about 42-50°C) followed by one or more washes in 0.3X SSC, at about 65-70°C. A preferred, non-limiting example of reduced stringency hybridization conditions includes hybridization in 4X SSC at about 50-60°C (or alternatively hybridization in 6X SSC plus 50% formamide at about 40-45°C) followed by one or more washes in 2X SSC, at about 50-60°C. Ranges intermediate to the above-recited values, e.g., at 65-70°C or at 42-50°C are

IV. Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors, for example recombinant expression vectors, containing an siRNA-encoding nucleic acid molecule. As used herein, the term 'vector' refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a 'plasmid', which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as 'expression vectors'. In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, 'plasmid' and 'vector' can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

In a preferred embodiment, the siRNA-encoding nucleic acid molecules are contained within an adenoviral vector, which can be used to infect mammalian cells (e.g., human cells). In a more preferred embodiment, the adenoviral vector is replication incompetent. For techniques related to adenovirus, see, inter alia, Felgner and Ringold (1989) Nature 337:387-388; Berkner and Sharp (1983) Nucleic Acids Res. 11:6003-6020; Graham (1984) EMBO J. 3:2917-2922; Bett et al. (1993) J. Virology 67:5911-5921; Bett et al. (1994) Proc. Natl. Acad. Sci. USA 91:8802-8806. A preferred adenoviral vector system is the AdEasy system (He, T.-C. et al. (1988) Proc. Natl. Acad. Sci. USA 95:2509-2514; Zeng, M. et al. (2001) Biotechniques 31:260-262).

Adenoviruses are non-enveloped, regular icosohedral, double-stranded DNA viruses. For a complete review on adenoviruses and their replication, see Horwitz, M. S., Virology 2d ed, Fields, B. N., eds., Raven Press Limited, New York (1990), Chapter 60, pp. 1679-1721. Publications describing various aspects of adenovirus biology and/or techniques relating to adenovirus include the following: PCT/US95/14461; Graham and Van de Eb (1973) Virology 52:456-467; Takiff et al. (1981) Lancet 2(8251):832-834; Berkner and Sharp (1983) Nucleic

Other suitable host cells are known to those skilled in the art. In a preferred embodiment, a host cell is a human cancer cell (e.g., a prostate cancer cell).

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques . As used herein, the terms 'transformation' and 'transfection' are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding an siRNA can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

In a most preferred embodiment, host cells containing the siRNAs of the invention are produced by infecting cells with a recombinant adenovirus containing the constructs.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents, and published patent applications cited throughout this application, as well as the figures and the sequence listing, are incorporated herein by reference.

Transfection of Cells

A total of 2 X 10^5 cells was seeded into each well of a six-well tissue culture plate (Falcon, Bedford, MA). The next day (when the cells were 70–80% confluent), the culture medium was aspirated, and the cell monolayer was washed with prewarmed sterile PBS. Cells were transfected with the appropriate construct using LipofectaminePlus reagent (Life Technologies) according to the manufacturer's protocol. Green fluorescence of pREV-transfected cells was quantified at each time point by FACS analysis and used to ascertain transfection efficiencies for cells transiently transfected with siRNA encoding plasmids.

FACS Analysis

For each sample, 1 X 10^4 cells were analyzed on a LSR flow cytometer (BD Biosciences, San Jose, CA) with an excitation wavelength of 488 nm and FITC collection wavelength using a band-pass filter at 530 ± 15 nm. Dead cells were gated out of the samples by forward and side scatter. The level of EGFP fluorescence in live cells was determined using the Becton Dickinson CellQuest program. FACS sorting ($\geq 10^2$ fluorescence on a four-log scale) was carried out at the same excitation/emission wavelengths using a BD FACS Vantage SE (BD Biosciences).

Clonogenic Survival

At the appropriate time after transfection, cells were trypsinized and diluted to the appropriate cell density into 100-mm culture dishes to give ≥50 colonies/dish after irradiation and then irradiated at 0.78 Gy/min to the desired dose using a Gammacell 40 ^{137}cesium irradiator (Atomic Energy, Ottawa, Canada). For MMS clonogenic assays, at the appropriate time after transfection, cells were treated for 1 hour with MMS solubilzed in DMSO and diluted in serum-free media (SFM) or an equivalent percentage of DMSO/SFM as a control, washed twice in PBS, and trypsinized and plated as explained above. Ten days after radiation or drug treatment, colonies comprising ≥50 cells were counted after staining with 50% Crystal Violet (Sigma- Aldridge, St. Louis, MO). Cell survival was plotted as a function of dose and fitted using the linear quadratic model $S = \exp.(-\alpha D - \beta D^2)$, where S is the cell survival, D is the dose of radiation, and α and β are constants. DRFs, the factor by which the dose of radiation or drug can be reduced in the presence of the sensitizing agent to achieve the same level of cell killing in the absence of the sensitizing agent, were calculated as the

closest to the translation initiation sequence (pATR-1 and pDNA-PK-1, respectively), whereas the two regions further downstream in the mRNA sequence gave the highest amount of protein inhibition for ATM (pATM-2 and pATM-3).

siRNA silencing of repair proteins renders human prostate tumor cells sensitive to DNA-damaging agents

Previous studies have demonstrated that loss of function of ATM, ATR, or DNA-PK_{cs} results in increased cellular sensitivity to DNA-damaging agents. To ascertain if siRNA-mediated attenuation of expression of ATM, ATR, and DNA-PKcs results in a subsequent sensitizing effect to such modalities, DU 145 and PC-3 human prostate cancer cells were transiently transfected with the ATM and DNA-PK_{cs}-targeted, siRNA encoding plasmids that were shown to give the greatest inhibition of target protein expression. At the appropriate time post-transfection where protein levels were shown to be the lowest, the resulting heterogeneously transfected cultures were treated with ionizing radiation. Cellular sensitivity was ascertained by clonogenic survival assays (Fig. 2). siRNA-mediated inhibition of these DNA repair proteins conferred an increased sensitivity to ionizing radiation in siRNA transfected cell populations compared with untransfected or pSHAG-transfected cells. This increased radiosensitivity corresponded to DRFs of 1.1 and 1.21 for DNA-PK_{cs} silencing in DU 145 and PC-3 cells, respectively, and 1.16 and 1.14 for ATM silencing in DU 145 and PC-3 cells, respectively, with an increase in sensitivity of ~1.5– 1.8-fold noted at 6 Gy. In addition, DU 145 cells transfected with ATR-targeted, siRNA-encoding plasmids exhibited an increased sensitivity (DRF = 1.38) to the alkylating agent MMS (Fig. 3). In a similar set of experiments, the expression of nontargeted siRNA in these cells failed to result in any evident radiation sensitization (DRF = 0.99). In addition, transfection of cells with siRNA plasmids that effectively reduced ATR protein levels also failed to enhance radiosensitivity (DRF = 0.98). Together, these data suggest that transfection and subsequent expression of plasmid-based siRNA does not, itself, result in an altered radiation response phenotype but actually requires specific targeting to produce such phenotypic alteration.

The biologically significant, but modest, degree of radiosensitization observed after transfection of ATM and DNA-PK_{cs} siRNA-encoding plasmids (Fig. 2) is a function of the heterogeneous populations resulting from transient transfection where the transfection efficiencies are ~25–40%. To enrich the transfected population and demonstrate a more representative clonogenic survival of the transfected/siRNA-expressing cells, DU 145 cells

plasmids were rendered sensitive to ionizing radiation (via targeting of ATM and DNA-PKcs) and the alkylating agent MMS (ATR).

Initial screening of siRNA for their effectiveness showed that all three target proteins were down-regulated by 90% from 24- to 72-h post-transfection (Figure 1), with protein levels being comparable with the levels seen in untransfected and vector-transfected cells at 96 h. These findings are consistent with previous data reporting the half-lives of these proteins to be in the region of 24–48 h (Lees-Miller, S. P. et al. (1996) J. Virol. 70:7471–7477; Fan, Z. et al. (2000) Cancer Gene Ther. 7:1307–1314).

The data presented herein demonstrate that siRNA-mediated inhibition in the expression of DNA repair proteins confers an increased sensitivity to therapeutically relevant DNA-damaging agents (Figs. 2 and 3). For ATM and DNA-PK_{cs}, siRNA-mediated attenuation of protein expression was manifested as an increased radiosensitivity for both heterogeneously transfected cells and FACS-enriched cells (respective DRFs of 1.15 and 1.4, with a 1.5–1.8- and 3-fold increase in radiosensitivity at 6 Gy in heterogeneously and transfected cells, respectively; Figs. 2 and 4). In addition, siRNA-mediated inhibition of ATR expression resulted in an increased sensitivity to the alkylating agent MMS (Fig. 3). Although DU 145 cells are somewhat resistant to alkylating agents because of a mutation in the mismatch repair protein hMLH1 (Chen, Y. et al. (2001) Cancer Res. 61:4112–4121; Yeh, C. C. et al. (2001) Biochem. Biophys. Res. Commun. 285:409–413), transfection of only 50% of cells with an ATR siRNA-encoding plasmid gave rise to an overall decreased cell survival (DRF of 1.38) after treatment with MMS (Fig. 3).

Although the observed increased radiation-mediated cell killing caused by DNA-PK_{cs} and ATM siRNA appears modest (average DRFs of 1.15; Fig. 2), it has to be emphasized that the survival curves shown represent a population of cells where only a minority (25–40%) are transfected and, thus, also have depleted levels of each target protein. To address this problem, DU 145 cells were cotransfected with the siRNA-encoding plasmid and a plasmid encoding EGFP, which were used to enrich the transfected population via FACS sorting. Western blot analysis showed that the FACS-sorted cells had a 80% reduction in both ATM and DNA-PK_{cs} expression (Fig. 4, A and D), which resulted in biologically significant DRFs of 1.46 and 1.36, with a 3-fold increased sensitivity noted at 6 Gy (Ref. 19; Fig. 4, B and E). Furthermore, the radiosensitivity exhibited by the enriched cells was greater than that seen in cells treated with the PI3k inhibitor Wortmannin or specific DNA-PKcs competitive inhibitor LY294002 (respective DRFs of 1.4 and 1.1; Fig. 4, C and F). The use of LY294002 before and after exposure to radiation highlights the increase in radiosensitivity

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

11. The method of claim 7, wherein the nitrosurea is selected from the group consisting of carmustine, lomustine, and streptozocin.

12. The method of claim 7, wherein the platinum complex is selected from the group consisting of carboplatin and cisplatin.

13. The method of claim 7, wherein the nonclassic alkylator is selected from the group consisting of altretamine, dacarbazine, procarbazine, and temozolamide.

14. The method of claim 4, further comprising contacting the cell with at least one chemotherapeutic agent.

15. The method of any one of claims 5-14, further comprising contacting the cell with at least a second chemotherapeutic agent.

16. The method of any one of claims 1-15, wherein the tumor cell is derived from a cell or tissue type selected from the group consisting of prostate, colon, breast, lung, brain, skin, ovary, pancreas, liver, stomach, bladder, bone, testicle, uterus, adipose tissue, throat, kidney, tongue, pituitary gland, thyroid, nerve, lymphoid tissue, eye, and cervix.

17. The method of any one of claims 1-16, wherein the tumor cell is resistant to killing by contacting with the DNA damaging agent alone.

18. The method of any one of claims 1-17, wherein the siRNA is expressed from a vector.

19. The method of claim 18, wherein the vector is a plasmid.

20. The method of claim 18, wherein the vector is an adenoviral vector.

21. The method of any one of claims 1-20, wherein expression of the siRNA is controlled by a modified adenoviral promoter.

32. The method of claim 29, wherein the alkyl sulfonate is busulfan.

33. The method of claim 29, wherein the nitrosurea is selected from the group consisting of carmustine, lomustine, and streptozocin.

34. The method of claim 29, wherein the platinum complex is selected from the group consisting of carboplatin and cisplatin.

35. The method of claim 29, wherein the nonclassic alkylator is selected from the group consisting of altretamine, dacarbazine, procarbazine, and temozolamide.

36. The method of claim 26, further administering to the subject a therapeutically effective amount of at least one chemotherapeutic agent.

37. The method of any one of claims 27-36, further administering to the subject a therapeutically effective amount of at least a second chemotherapeutic agent.

38. The method of any one of claims 23-37, wherein the cancer is derived from a cell or tissue type selected from the group consisting of prostate, colon, breast, lung, brain, skin, ovary, pancreas, liver, stomach, bladder, bone, testicle, uterus, adipose tissue, throat, kidney, tongue, pituitary gland, thyroid, nerve, lymphoid tissue, eye, and cervix.

39. The method of any one of claims 23-37, wherein the cancer is resistant to treatment by administration of the DNA damaging agent alone.

40. The method of any one of claims 23-39, wherein the siRNA is expressed from a vector.

41. The method of claim 40, wherein the vector is a plasmid.

42. The method of claim 40, wherein the vector is an adenoviral vector.

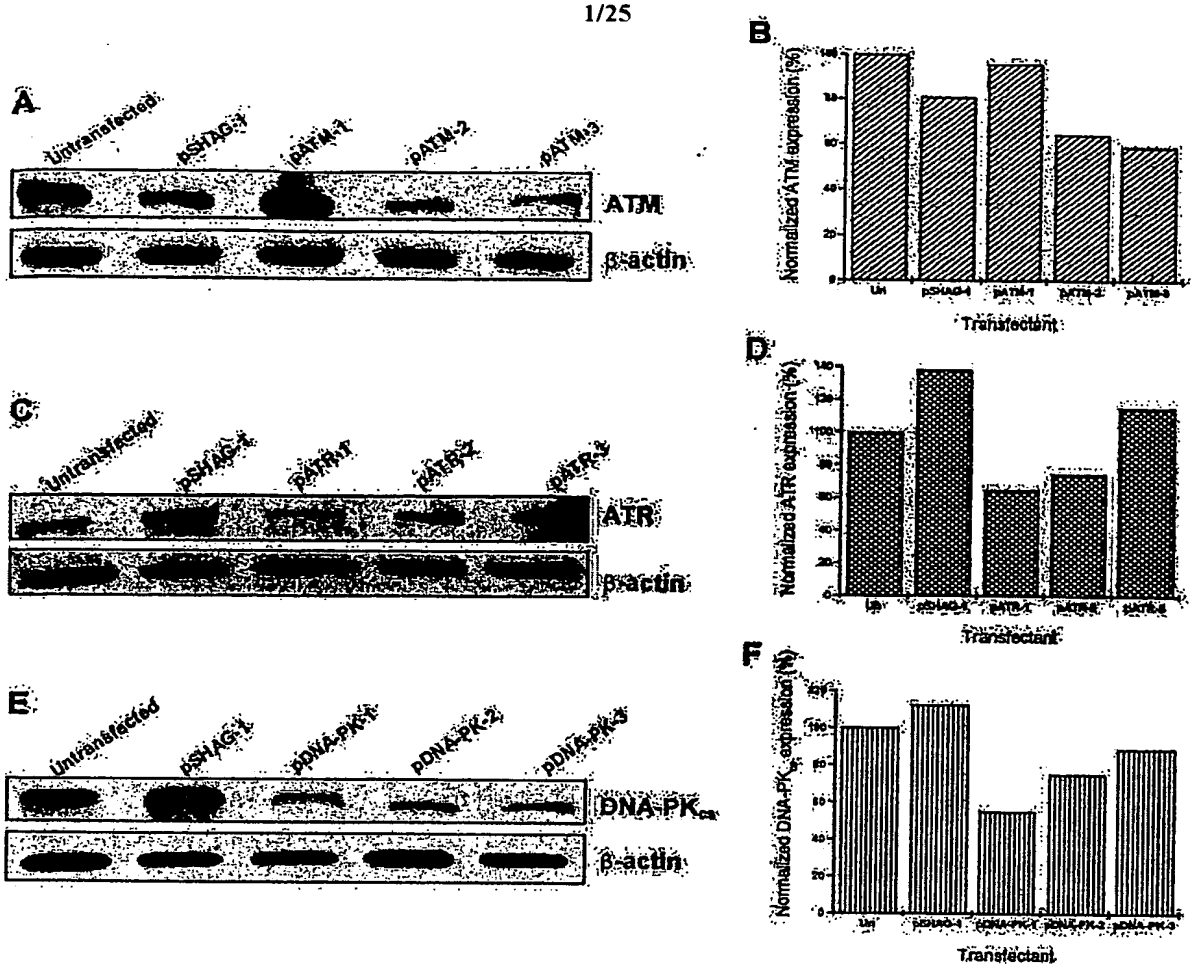

A
Untransfected
pSHAG-1
pATM-1
pATM-2
pATM-3
ATM
β-actin
B
Normalized ATM expression (%)
Transfectant
C
Untransfected
pSHAG-1
pATR-1
pATR-2
pATR-3
ATR
β-actin
D
Normalized ATR expression (%)
Transfectant
E
Untransfected
pSHAG-1
pDNA-PK-1
pDNA-PK-2
pDNA-PK-3
DNA-PKcs
β-actin
F
Normalized DNA-PKcs expression (%)
Transfectant

Fig. 3

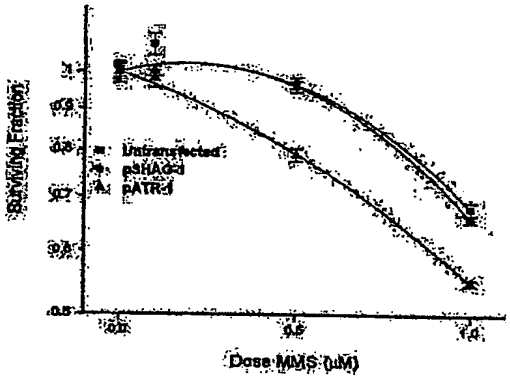

Surviving Fraction
Untransfected
pSHAGE-I
pATR-I
0.5
0.6
Dose MMS (μM)

Fig. 5

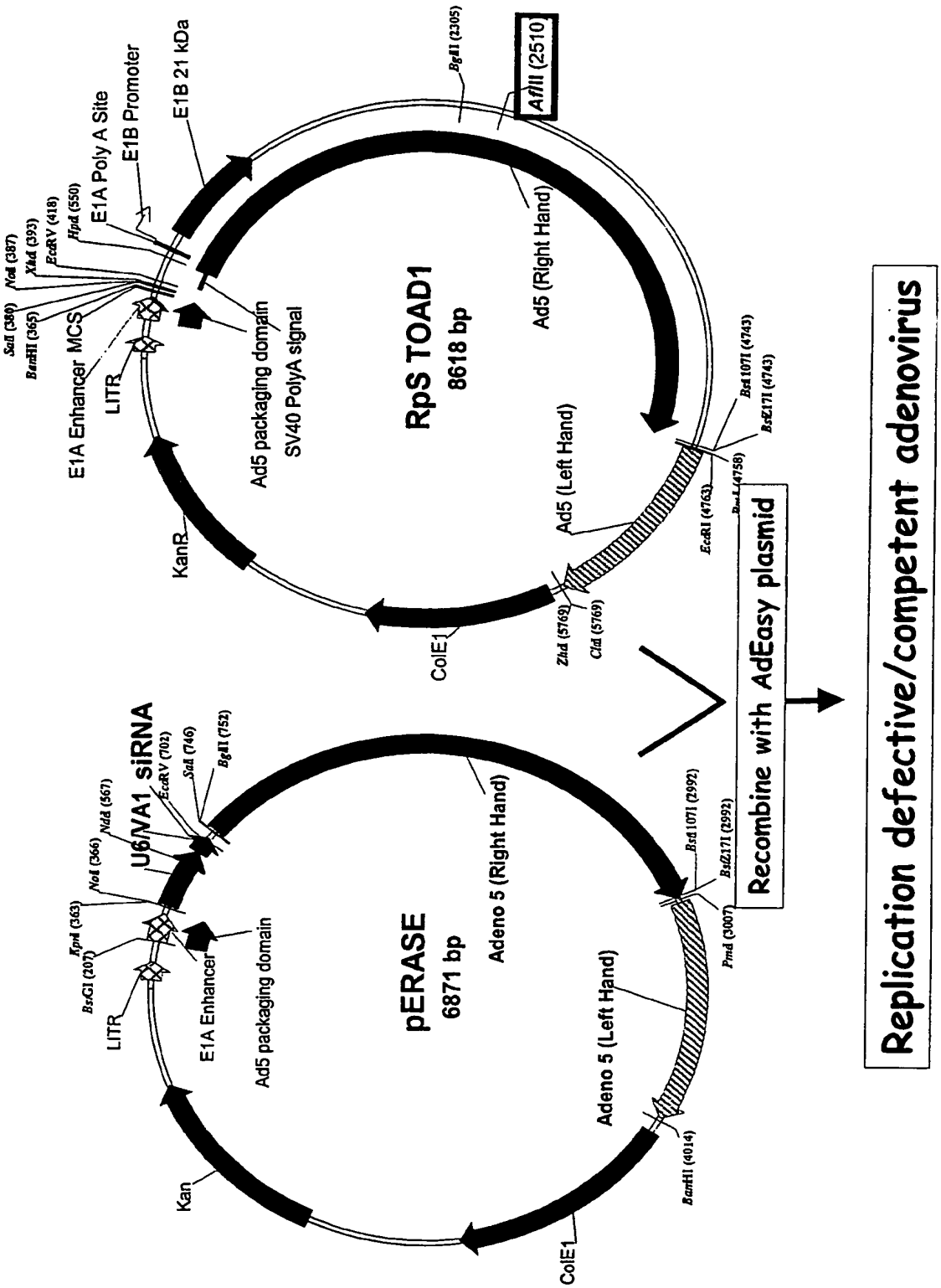


Fig. 7

Effectiveness of VA1 promoter in PC-3 Luc cells

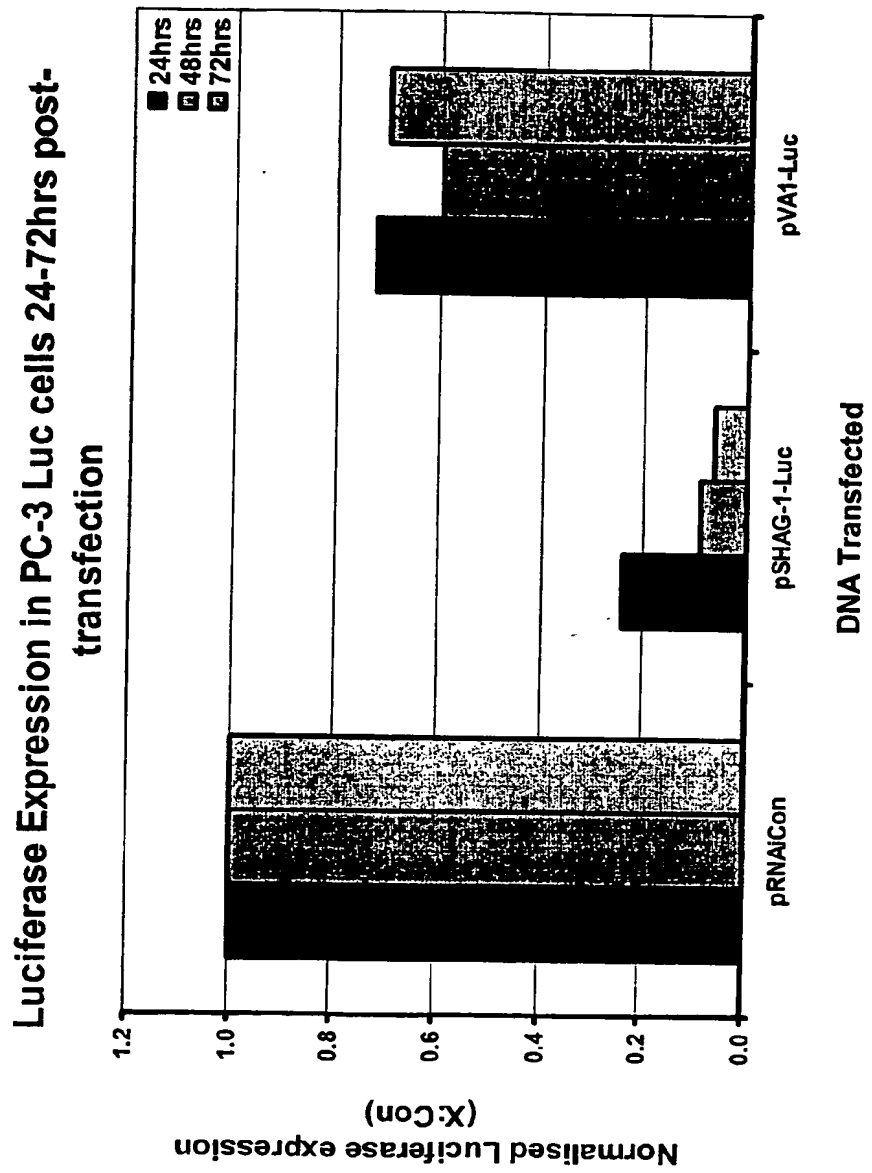


Fig. 9

Effectiveness of VA1 promoter in DU 145 cells: co-transfection experiments

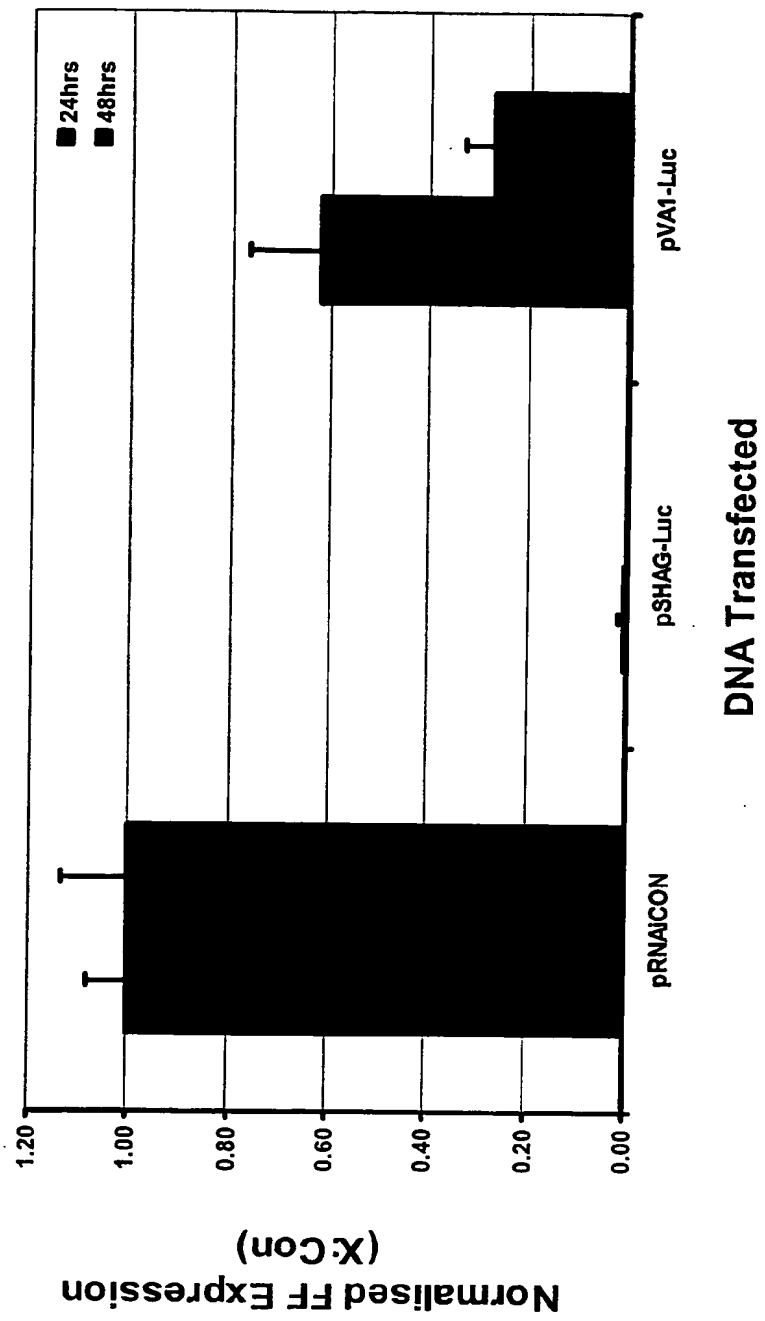


Fig. 11

VA1 versus U6-mediated down-regulation of DNA-PK in DU 145 cells

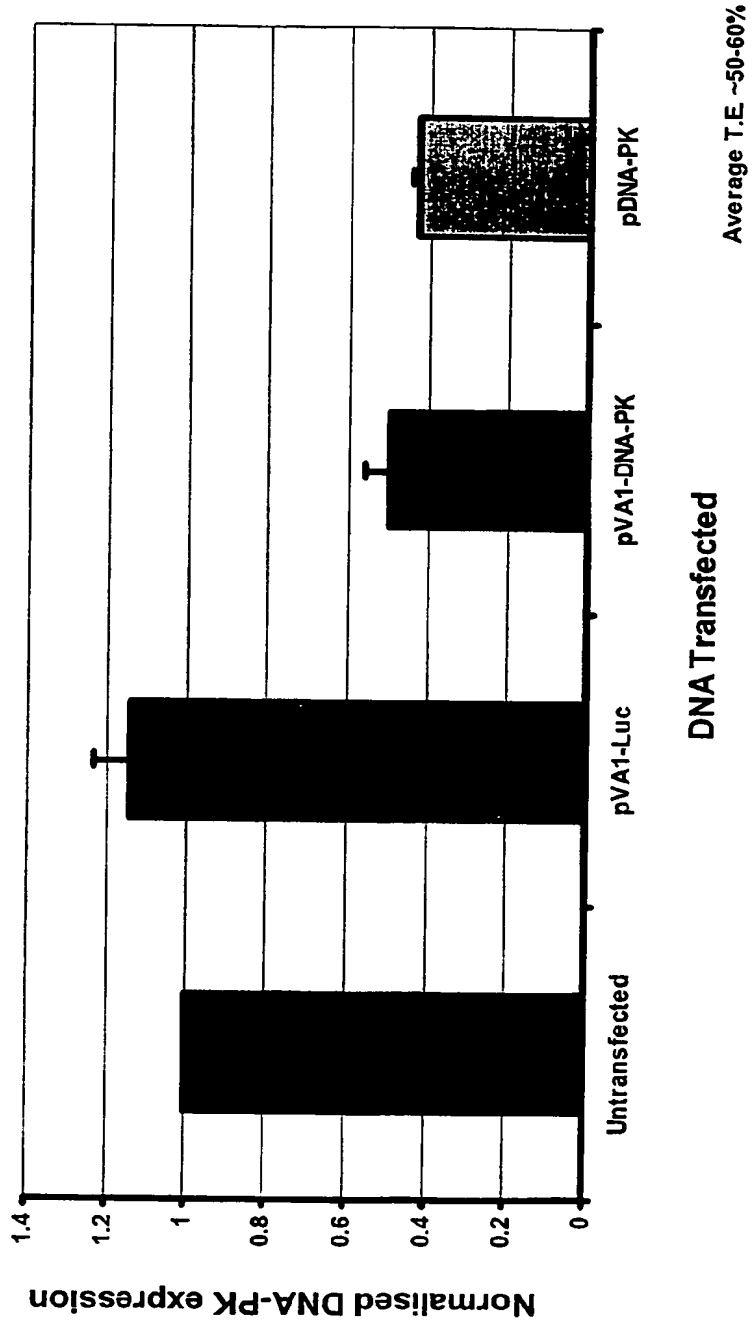
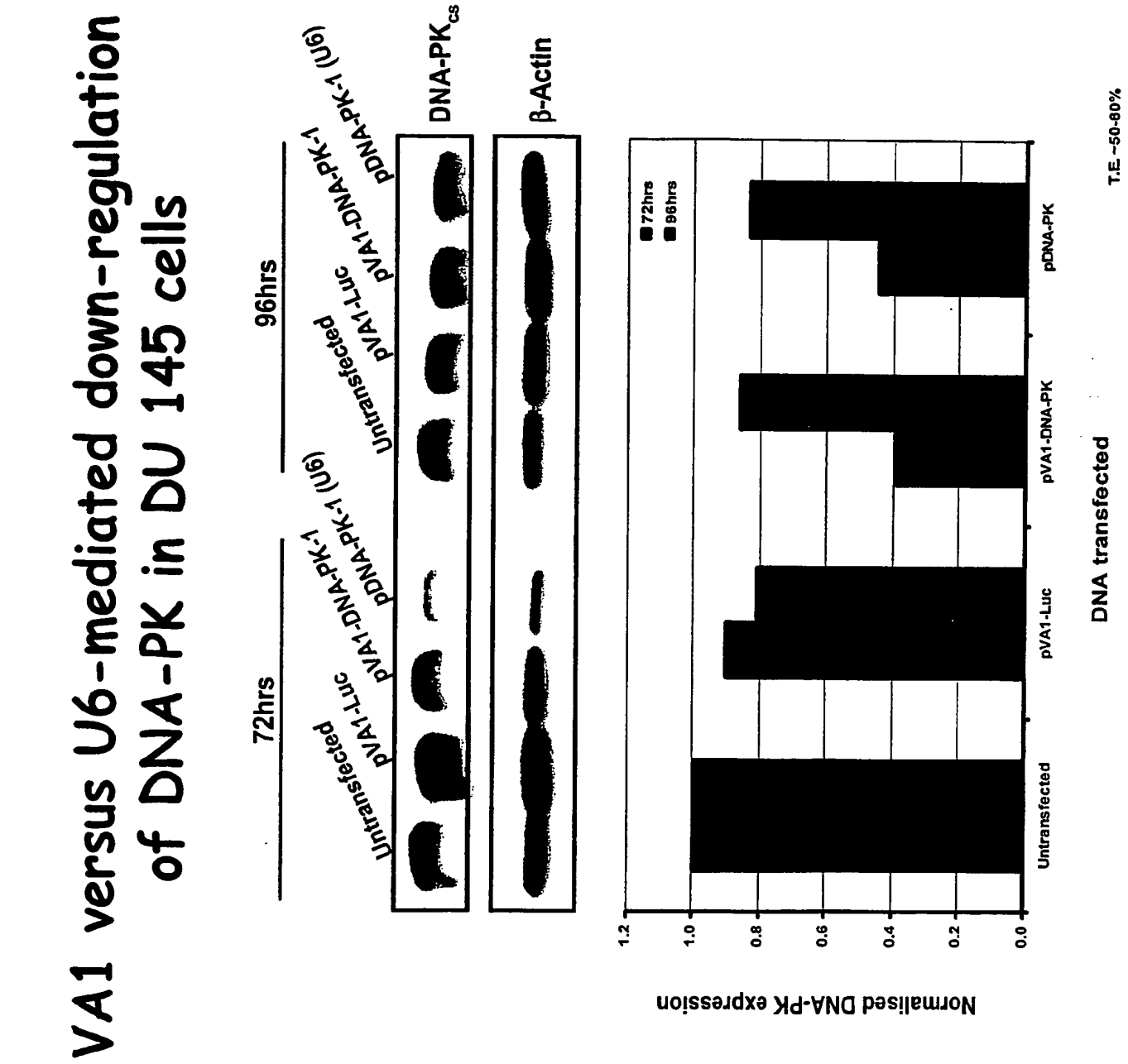


Fig. 13



ATM-2 (5'- 3')

Oligo-A

CCTGGAGGCTTGTGTTGAGGCTGATACAGAAGCTTGTGTATCAGCCTCAGCATAAGCCTCCGGGTAGTTTTTT

Oligo-B

GATCAAAAAACTACCCGGAGGCTTATGCTGAGGCTGATACACAAGCTTCTGTATCAGCCTCAACACAAGCCTCCAGGCG

Target sequence:-

TGTATCAGCCTCAACACAAGCCTCCAGGCAG (432-462, ATG = 190)

G/C content = 55%

Fig. 15

ATR-1 (5'- 3')

Oligo-A

TATTATATTCCTCTGGTGTGGCACTGCCGAAGCTTGGGCAGTGTCACACTAGAGGGATATAGTACAGTTTTTT

Oligo-B

GATCAAAAAACTGTACTATATCCCTCTAGTGTGACACTGCCCAAGCTTCGGCAGTGCCACACCAGAGGAATATAATACG

Target sequence:-

GGCAGTGCCACACCAGAGGAATATAATACAG (134-164, ATG = 80)

G/C content = 48%

Fig. 17

ATR-3 (5'- 3')

Oligo-A

CTCATGACCACTGGCCATTCCACAGCATGAAGCTTGATGCTGTGGAGTGGCCGGTGGTTATGAGTCGTTTTTT

Oligo-B

GATCAAAAAACGACTCATAACCACCGGCCACTCCACAGCATCAAGCTTCATGCTGTGGAATGGCCAGTGGTCATGAGCG

Target sequence:-

ATGCTGTGGAATGGCCAGTGGTCATGAGCCG (579-609, ATG = 80)

G/C content = 58%

Fig. 19

DNA-PK-2 (5'- 3')

Oligo-A

GATGAACTTCACCCAATAATCCTAGGAGGAAGCTTGCTTCTAGGATTATTGGGTGGAGTTCGTCTTATTTTTT

Oligo-B

GATCAAAAAATAAGACGAACTCCACCCAATAATCCTAGAAGCAAGCTTCCTCCTAGGATTATTGGGTGAAGTTCATCCG

Target sequence:-

CTCCTAGGATTATTGGGTGAAGTTCATCCTA (585-616, ATG = 58)

G/C content = 42%

Fig. 21

ATM (5'- 3')

TAGCTCTATCATGTTCTAGTTGACGGCAX$_n$TGCCGTCGACTAGGACATGGTAGAGTTACAGTTTTTT

CCTGGAGGCTTGTGTTGAGGCTGATACAX$_n$TGTATCAGCCTCAGCATAAGCCTCCGGGTAGTTTTTT

TAGTATGTTGCTACAATCAGCTCCGTAAX$_n$TTACGGAGCTGATTGTGGCGACGTATTACTCTTTTTT

ATR (5'- 3')

TATTATATTCCTCTGGTGTGGCACTGCCX$_n$GGCAGTGTCACACTAGAGGGATATAGTACAGTTTTTT

TTGCTGCAATCCGCAGAAGTCTCGTTATX$_n$ATAATGAGACTTCTGCGGATTGTAGTAATTCTTTTTT

CTCATGACCACTGGCCATTCCACAGCATX$_n$ATGCTGTGGAGTGGCCGGTGGTTATGAGTCGTTTTTT

DNA-PK (5'- 3')

ATGTCTGTAATGCCAGCACCGCGGGGCTX$_n$AGCCTCGTGGTGCTGGTATTACAGATATCTTTTTTTT

GATGAACTTCACCCAATAATCCTAGGAGX$_n$CTTCTAGGATTATTGGGTGGAGTTCGTCTTATTTTTT

TGAAGTTGCACAGAAGTGAGGACAACCCX$_n$GGGTTGTTCTTACTTCTGTGCAGCTTCATTATTTTTT

Fig. 23

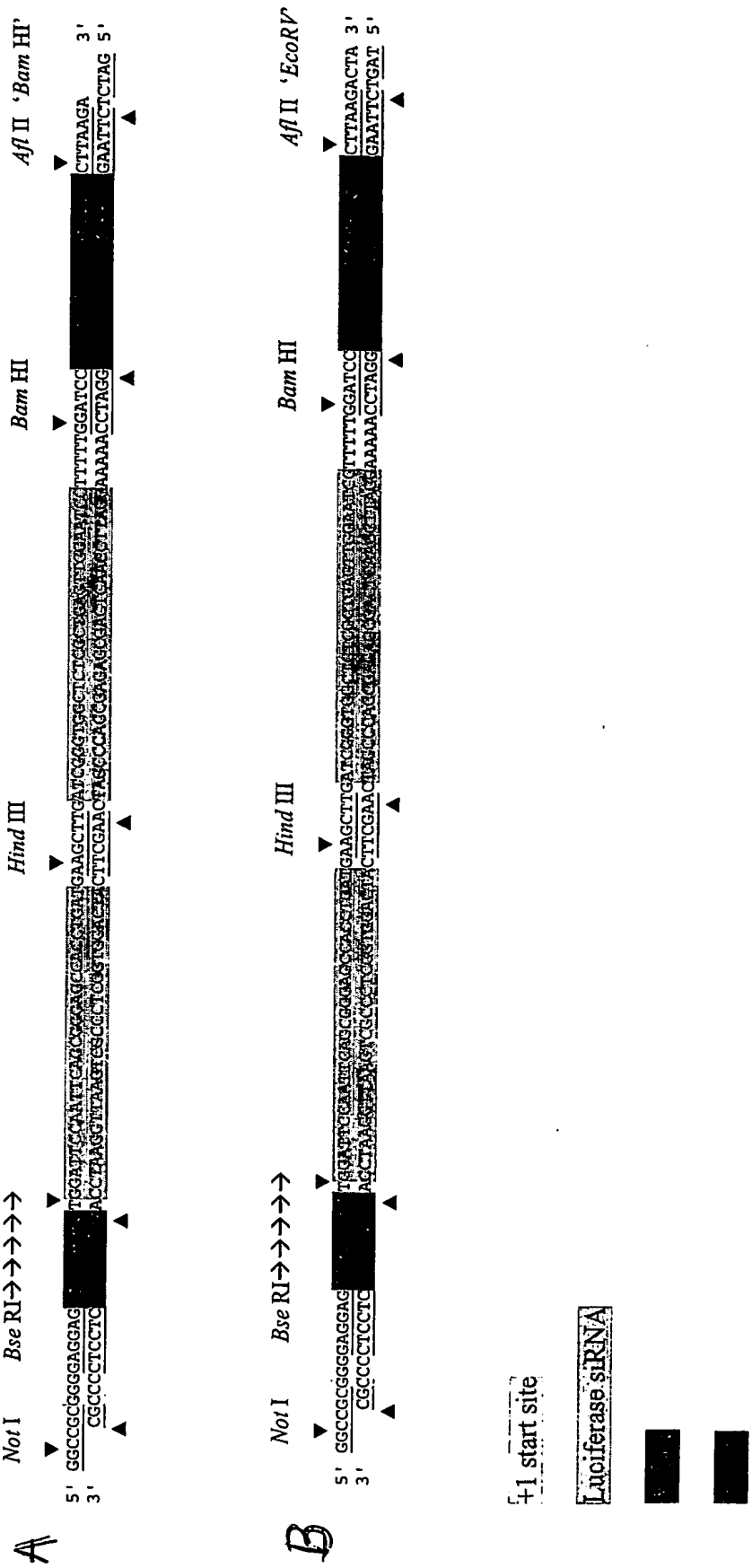


Fig. 25